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We sought to assess the importance of cooperative interactions between p185^{erbB-2} and *erb*B-3 in the growth factor-independent proliferation and neoplastic transformation of breast carcinoma cells with c-*erb*B-2 gene amplification. We have now successfully produced cell populations derived from MCF-10A normal mammary epithelial cells that overexpress c-*erb*B-2 at very high levels comparable to that seen in breast carcinoma cells with c-*erb*B-2 gene amplification. While the original clones of MCF-10A*erb*B-2 cells overexpress c-*erb*B-2 at only moderate levels and are not tumorigenic in nude mice, MCF-10A*erb*B-2shH cells selected for high-level c-*erb*B-2 overexpression with growth factor independence in culture show a high level of constitutive PI 3-kinase activity and a highly transformed phenotype both in culture and *in vivo*. In addition, key observations were made during the course of these studies which provide important information concerning the survival and growth of cells in the absence of growth factors that occurs as a function of the level of c-*erb*B-2 gene overexpression. Experiments are continuing to study the changes in the levels of p185^{erbB-2}/p185^{erbB-2} and p185^{erbB-2}/*erb*B-3 constitutive activation seen in these cell lines, and to relate the accompanying changes in PI 3-kinase activation (and other effects of c-*erb*B-2 overexpression) to the threshold level of signaling that is sufficient to induce the effective transformation of MCF-10A cells. Also, the use of recently constructed bicistronic retroviral expression vectors containing a dominant negative form of c-*erb*B-3 will allow us to test the relative importance of p185^{erbB-2}/*erb*B-3 heterodimer function in various cell lines.

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Stephen P. Ethier 6/25/98

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INTRODUCTION

We sought to assess the importance of the cooperative interactions that occur between p185^{erbB-2} and *erb*B-3 receptor tyrosine kinases during the growth factor-independent proliferation and neoplastic transformation of breast carcinoma cells with c-*erb*B-2 gene amplification. The c-*erb*B-2 (*neu*/HER-2) gene encodes an 185 kDa protein tyrosine kinase that is highly homologous to the epidermal growth factor receptor (EGFR/*erb*B- 1), *erb*B-3 (HER-3) and *erb*B-4 (HER-4) (1-3), which together, comprise the type 1 family of receptor tyrosine kinases (4, 5). The *erb*B receptor tyrosine kinases all contain ectodomains with two cysteine-rich sequences. Despite this structural homology, these receptors differ in their ligand specificities (4). Thus, EGFR binds several distinct ligands (e.g. transforming growth factor-α, amphiregulin, heparin-binding EGF and betacellulin) whose prototype is EGF, whereas *erb*B-3 and *erb*B-4 are the respective low and high affinity receptors for more than a dozen different isoforms of *neu* differentiation factor/heregulin (HRG) (6-8). While no direct ligand for p185^{erbB-2} has yet been cloned, it is now clear that p185^{erbB-2} is capable of heterodimerization with EGFR (9, 10), *erb*B-3 (11) or *erb*B-4 (8), and these p185^{erbB-2}-containing heterodimers form the highest affinity binding sites for their respective ligands (10, 11). c-*erb*B-2 is amplified in 28% of primary breast carcinomas *in vivo* (12) and another 10% overexpress c-*erb*B-2 without amplification of the gene (14-16). In addition, c-*erb*B-2 gene amplification, concordant with high-level overexpression, is correlated with increased tumor aggressiveness and the poor prognosis of breast cancer patients (13, 14, 17-20). Other related genes, such as the EGFR gene, are sometimes amplified in human breast cancers (13). However, amplification of the EGFR gene is much less common than that seen for c-*erb*B-2 (2% vs 28%, respectively) in breast cancer. While amplification of c-*erb*B-3 or c-*erb*B-4 was not seen in various studies (2, 3), our own work and others have now shown that heterodimer interactions between p185^{erbB-2} and *erb*B-3 are constitutively activated in breast cancer cells with c-*erb*B-2 gene amplification (21-23), and co-transfection of c-*erb*B-3 with c-*erb*B-2 greatly augments the transforming capability of c-*erb*B-2 (22). These p185^{erbB-2}/*erb*B-3 heterodimer complexes activate the mitogen-activated protein (MAP)-kinase and phosphatidylinositol (PI) 3-kinase signal transduction pathways. By constitutively activating key signal transduction pathways to a level that is effective for transformation, tumor cells apparently escape the normal controls on cell cycle regulation. Also, much recent evidence suggests that a *critical threshold* level of c-*erb*B-2 gene overexpression is required to effectively transform cells. We are particularly interested in how high the level of c-*erb*B-2 gene expression must be to constitutively activate key signal transduction pathways that fully transform human mammary epithelial cells, and how the cooperative effects of p185^{erbB-2}/*erb*B-3 heterodimer action affects this critical threshold.

Experimentally elevated c-*erb*B-2 gene expression in various cell lines (24-27), including non-transformed human mammary epithelial cells (27), induces the complete transformation of cells injected into nude mice. The potent oncogenic potential of p185^{erbB-2} is generally thought to be due to its ability to constitutively activate different key signal transduction pathways that are involved in the regulation of cell growth. While our current understanding of the oncogenic potential of p185^{erbB-2} has expanded quite rapidly (for review see 28), our knowledge of how exactly p185^{erbB-2} induces full neoplastic transformation in human mammary epithelial cells still remains fragmentary. For example, although p185^{erbB-2} was originally discovered as the *neu*

transmembrane-mutated form of the gene in rat neuroblastoma cells (29), the c-*erb*B-2 gene found in human breast cancer cells has never shown such mutations (30). However, the level of of tyrosine-phosphorylated p185^{erbB-2} in primary human mammary carcinomas *in vivo* always shows a direct correspondence with the overexpression of p185^{erbB-2} (31), suggesting that overexpression of wild-type c-*erb*B-2 alone is sufficient to constitutively activate its tyrosine kinase function. Furthermore, the protein encoded by the wild-type c-*erb*B-2 gene was previously shown to exhibit constitutive tyrosine kinase activity if sufficiently overexpressed in a variety of cell lines in culture in the absence of any identifiable ligand (24-27, 32, 33). Also, transfection of a gene encoding a chimeric receptor containing the EGFR extracellular domain fused to the cytoplasmic domain of p185^{erbB-2} results in the constitutive tyrosine kinase activity of the chimeric receptor in the absence of EGF (32, 33), indicating that the tyrosine kinase domain of p185^{erbB-2} exhibits a greater tendency towards ligand-independent activation than does the EGFR tyrosine kinase domain when overexpressed in various cell types. Therefore, the constitutive activation of p185^{erbB-2} in breast carcinoma cells is generally thought to be due to the high-level overexpression of the wild-type protein, whose activity shows a strong propensity towards ligand-independent activation when overexpressed (28, 32, 33). However, some ambiguity still exists concerning a possible role for the heregulins (ligands which activate p185^{erbB-2} through direct binding to *erb*B-3 or *erb*B-4), and/or other putative ligands of p185^{erbB-2} in the constitutive activation of p185^{erbB-2} in transfected cell lines and tumor cells.

Another area of great importance concerns the heterodimeric associations which have been recently shown to occur between the different type 1 receptor tyrosine kinases, including EGFR and p185^{erbB-2} (9, 10), p185^{erbB-2} and *erb*B-3 (11, 12), p185^{erbB-2} and *erb*B-4 (8), and EGFR and *erb*B-3 (34, 35). These heterodimeric interactions may occur through both ligand-dependent and ligand-independent mechanisms. Activation of tyrosine kinase activity of these receptors involves dimerization, trans-autophosphorylation and the recruitment of various signal transduction molecules by specific tyrosine-phosphorylated receptor carboxyl-terminal sites (that are different for the various *erb*Bs). Thus, differential responses mediated by the various erbBs and their respective ligands can become quite complex, because of the various combinatorial associations between the different receptors and the relative affinities between different receptor dimers at various levels of expression. The possible interactions between the different *erb*B receptors has only very recently emerged as an important concept in *erb*B-2-related cancer biology. As mentioned above, our own work and others have now shown that the heterodimer interactions between p185^{erbB-2} and *erb*B-3 are constitutively activated in breast cancer cells with c-*erb*B-2 gene amplification (21-24). With regard to the activation of the PI 3-kinase signaling pathway, *erb*B-3 is known to contain 7 potential YXXM phosphorylation sites that, when tyrosine-phosphorylated, form PI 3-kinase docking sites within the cytoplasmic region of the receptor (5). In contrast, the other *erb*Bs only contain one such docking site. Therefore, the cooperative interactions between p185^{erbB-2} and *erb*B-3 that mediate the constitutive activation of various signaling pathways, including PI 3-kinase, has come center-stage for understanding the autonomous growth of cancer cells with c-*erb*B-2 gene amplification. The cooperative interactions between the different receptor tyrosine kinases may help explain the pleiotropic effects of p185^{erbB-2} on multiple signaling pathways which lead to the full transformation of cells. Furthermore, by studying these interactions, we can better devise new potential targets for

clinical intervention employing gene therapy and other methods to block constitutive p185$^{erbB\text{-}2}$ activity in cancer cells.

Our laboratory is interested in studying mechanisms of growth factor dependency in mammary epithelial cells. We have previously shown that the growth factor-independent proliferation of mammary carcinoma cells in culture is consistently associated with their malignant potential when serially transplanted *in vivo* (36). Both autocrine (37) and non-autocrine (38) mechanisms may be involved in these phenomena, depending on the particular tumor and stage of the disease. Other studies have also shown a correlation of growth factor-independent proliferation in culture with tumor metastasis *in vivo* (39). Therefore, growth factor independence, as a phenotype, is a good indicator of progressive cell transformation in tumor cells. Normal human mammary epithelial cells require both insulin-like growth factor (IGF)-l (or supra physiological levels of insulin) and EGF to proliferate under serum-free conditions in culture (40-42). The synergistic requirement for both IGF and EGF during the mitogen-dependent proliferation of normal mammary epithelial cells suggests that the attainment of full growth factor independence in mammary carcinoma cells involves genetic changes which subvert the normal requirements for both IGFs and EGF during mammary epithelial cell proliferation. We have recently shown that 21MT human breast carcinoma cell lines, that overexpress progressively elevated levels of c-*erb*B-2 (see original proposal), exhibit IGF independence at moderate levels of c-*erb*B-2 overexpression, and combined IGF/EGF independence at the highest level of c-*erb*B-2 gene overexpression (43). Furthermore, we found that the HRGs are mitogenic for human mammary epithelial cells (which express both c-*erb*B-2 and c-*erb*B-3, but not c-*erb*B-4) in the absence of IGF and EGF in culture (43, 44). Thus, activation of p185$^{erbB\text{-}2}$/*erb*B-3 heterodimers can mimic the combined actions of both IGF and EGF in mammary epithelial cells. This indicates that the combined activation of p185$^{erbB\text{-}2}$ and *erb*B-3 (either constitutively or in the presence of exogenous HRG) mediates the activation of signal transduction mechanisms which substitute for IGF and EGF during mammary epithelial cell proliferation. The ability of various growth factors to stimulate normal cell growth was previously shown to fall into two different functional categories termed competence (e.g. EGF) and progression (e.g. IGF-1) factors (45). EGF is required during the early part of the G1 phase of the cell cycle to make the cells competent for proliferation, while IGF is required towards the end of G1 for the cell to progress into S phase of the cycle, and both EGF and IGF are required transiently during the middle of G1 (45). Therefore, these different growth factors act synergistically to stimulate proliferation in a fashion that is not simply additive, but is indicative of synergism between growth factor responsive pathways which must be activated together for mitogenesis in non-transformed cells that express normal receptor levels. Interestingly, much of the work concerned with understanding how oncogene expression subverts these growth factor requirements in tumor cells has emphasized responses associated with the requirement for competence factors, because other oncogenes, such as v-*erb*B (a truncated form of EGFR), were only implicated in the activation of progression pathways that normally require EGF for stimulation (46). We propose that p185$^{erbB\text{-}2}$ constitutive activation in mammary carcinoma cells substitutes for growth factor-mediated signal transduction pathways normally requiring the combination of both IGF and EGF, and this autonomous growth potential depends on the level of p185$^{erbB\text{-}2}$ activation in specific tumor cell populations as well as the interaction of p185$^{erbB\text{-}2}$ with other type 1 receptor tyrosine kinases. Finally, all of these complex properties of p185$^{erbB\text{-}2}$

may help explain the common occurrence and selection of cells with c-*erb*B-2 gene amplification that is seen in certain cancers, such as breast and ovarian carcinoma.

Our data, in addition to others, now strongly implicate PI 3-kinase-mediated signal transduction in HRG-induced mitogenesis, as well as constitutively in the autonomous growth of breast carcinoma cells with c-*erb*B-2 gene amplification (22, 23). However, further work is required to determine the minimum level of c-*erb*B-2 gene overexpression that is required for constitutive signal transduction, growth factor independence, and tumorigenicity of mammary epithelial cells that commonly co-express c-*erb*B-3. Previous studies have shown that experimentally elevated c-*erb*B-2 gene expression in non-neoplastic human mammary epithelial cells may be sufficient to convert the cells to a full neoplastic phenotype when injected into nude mice (27). Interestingly, it was also reported that the growth of the transfected heterogenous cell populations in nude mice had much greater elevation of c-*erb*B-2 gene expression in tumor cells which grew out *in vivo* then did the original transfectants, and this higher level of c-*erb*B-2 overexpression correlated with increased soft agarose growth in culture and increased tumor growth *in vivo* in a manner which suggested to us that the selection of variants containing higher levels of c-*erb*B-2 expression was required to induce full transformation (27). However, it was earlier reported that c-*erb*B-2 overexpression alone was not sufficient to fully transform MCF-10A normal mammary epithelial cells (47). But the MCF-10A*erb*B-2 cell clones that were derived in those studies only showed moderate levels of c-*erb*B-2 gene expression relative to tumor cells with c-*erb*B-2 gene amplification (unpublished results; see below). Additionally, another cell line derived from the primary tumor of a patient with breast carcinoma containing c-*erb*B-2 gene amplification (48, 49) shows amplification of c-*erb*B-2, but shows much lower levels of gene expression and constitutive $p185^{erbB-2}$ activation then does the metastatic tumor cell lines from the same patient (manuscript in preparation). This 21PT (primary tumor) cell line also does not show as high a level of growth factor independence in culture or form tumors in nude mice *in vivo*, while the 21MT (metastatic tumor) cell lines do (49). This also supports the contention that there is a critical threshold level of c-*erb*B-2 gene overexpression (i.e. between that seen in 21PT and 21MT cells) that is necessary to effectively transform human mammary epithelial cells. Furthermore, all of the cell lines mentioned above co-express c-*erb*B-3, but not c-*erb*B-4 (43, 44). The minimum level of c-*erb*B-2 gene overexpression that is required for full transformation in mammary epithelial cells, which routinely co-express c-*erb*B-3, has not yet been precisely determined. In addition, none of the above studies with transfected mammary cells have measured the signal transduction events which underlie these phenomena in experimentally generated c-*erb*B-2-overexpressing human mammary epithelial cells. Therefore, we have begun a series of experiments to address these questions by producing c-*erb*B-2-overexpressing mammary epithelial cell lines by infection with retroviral expression vectors combined with procedures for the selection of cell populations that overexpress progressively elevated levels of $p185^{erbB-2}$ truly comparable to that seen in tumor cells with c-*erb*B-2 gene amplification. Using these vectors and cell lines, we can now complete our objectives to determine the critical threshold level of c-*erb*B-2 overexpression and constitutive activation that is required to effectively transform various normal human mammary epithelial cell lines.

In this project, we have also focused on specific strategies for blocking $p185^{erbB-2}$/*erb*B-3 action in breast carcinoma cells with c-*erb*B-2 gene amplification. The relative importance of the $p185^{erbB-2}$-*erb*B-3 interaction (relative to $p185^{erbB-2}$/$p185^{erbB-2}$ homodimer

function) in the constitutive activation of PI 3-kinase and growth factor independence of tumor cells with c-*erb*B-2 gene amplification has not yet been clearly established. One strategy which has been used successfully for other receptor tyrosine kinases employs dominant negative vectors, wherein the region coding for the cytoplasmic region of the receptor is almost completely removed (Fig. 1). While the mutant truncated receptor still contains the transmembrane domain and can thus dimerize within the cell, it lacks tyrosine kinase activity and inhibits the signal transduction docking function of both the wild-type and mutant receptors within heterodimers. Therefore, by specifically removing this region the receptor function is completely impaired. This strategy has been used effectively for blocking EGFR (50), platelet-derived growth factor receptor (51), fibroblast growth factor receptor (52), and *neu* (53) function in various studies. In order to block the constitutive activation of $p185^{erbB-2}$/*erb*B-3 in mammary carcinoma cells, as well as the HRG-induced activation of $p185^{erbB-2}$/*erb*B-3, we are in the processess of constructing expression vectors that code for dominant negative forms of *erb*B-3 and $p185^{erbB-2}$ for introduction into various cell lines. The introduction of these dominant negative vectors in cells will allow us to better assess the relative importance of $p185^{erbB-2}$/*erb*B-3 heterodimer function in the autonomous growth of carcinoma cells with c-*erb*B-2 gene amplification, as well as the effects of HRGs in various cell types.

BODY

Derivation of MCF-10A*erb*B-2 Cells with High-level Overexpression of $p185^{erbB-2}$, IGF Independence and a Fully Transformed Phenotype

In order to determine the level of c-*erb*B-2 gene overexpression that is required for mammary epithelial cells to constitutively activate PI 3-kinase, induce growth factor independence and undergo transformation, it was necessary to genetically engineer new mammary epithelial cell lines that overexpress c-*erb*B-2 at very high levels comparable to that seen in breast carcinoma cells with c-*erb*B-2 gene amplification. As discussed above, previous studies involving genetically engineered MCF-10A cells were not successful in generating cell populations which sufficiently overexpress c-*erb*B-2 at levels comparable to that seen in breast carcinoma cells with c-*erb*B-2 gene amplification, and this may explain the lack of full transformation in MCF-10A*erb*B-2 cells used in previous studies. Thus, it is of primary importance to develop new $p185^{erbB-2}$-overexpressing cell lines for a more critical assessment of threshold levels of c-*erb*B-2 gene overexpression required for transformation in human mammary epithelial cells that co-express c-*erb*B-3 (see original proposal for more details). To accomplish the initial specific aims of this project, we constructed a bicistronic retroviral vector containing the c-*erb*B-2 gene under the control of the CMV promoter (see original proposal). We also utilized previously constructed cell lines which overexpress c-*erb*B-2 to select for high-level overexpressing cell populations using Flow Cytometry (see previous progress report). The original pSLH*erb*B-2 vector we produced was constructed by blunt-end ligation of the full-length c-*erb*B-2 cDNA into the pSLH1001 retroviral expression vector. However, transfection and infection of cell lines with this vector did not show increased expression of c-*erb*B-2. We have previously used the pSLH1001 vector successfully. Therefore, it was likely that alterations

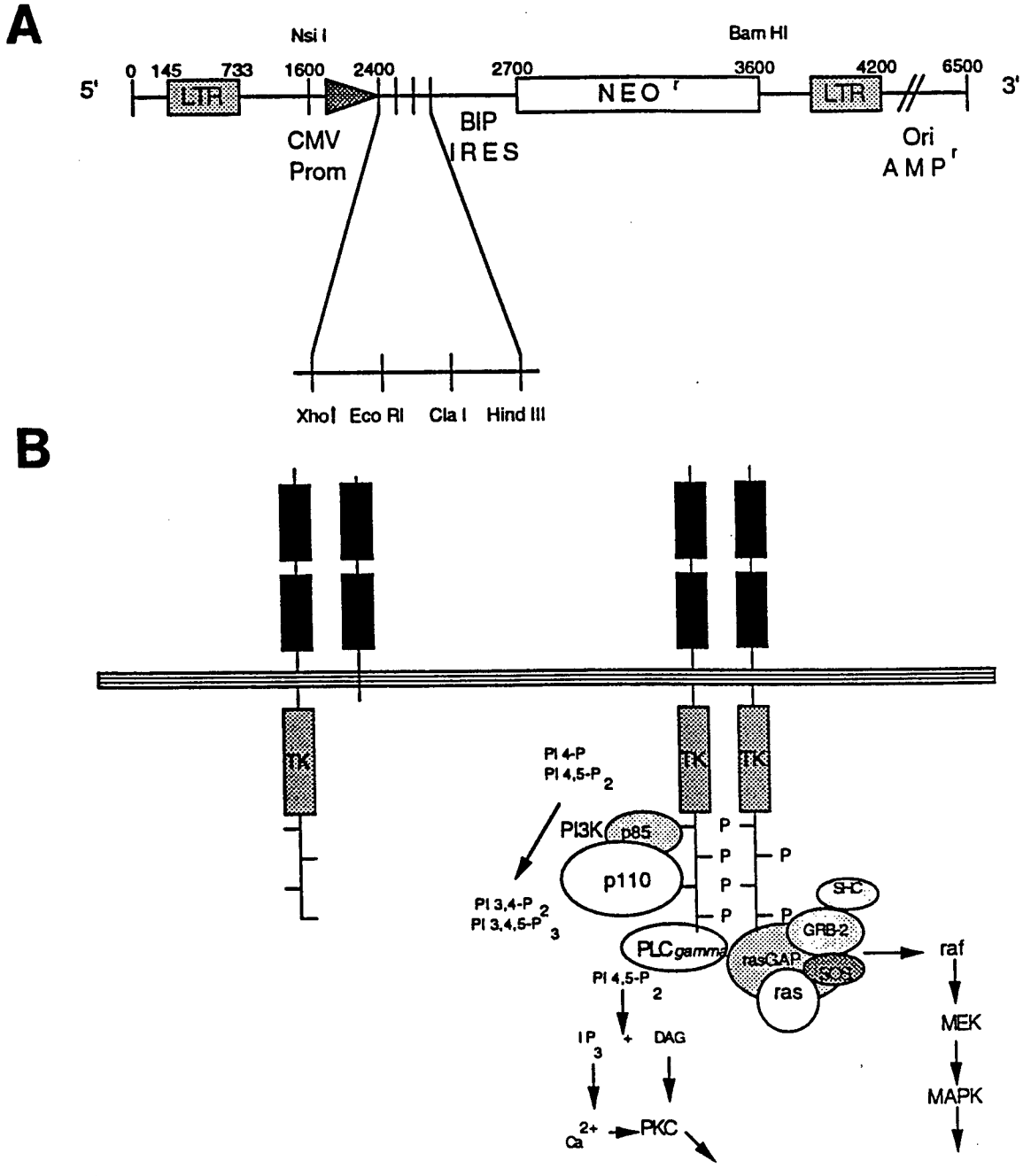


Fig. 1. ***Expression vectors for use in this study.*** (A) The pSLH1001 bicistronic retroviral expression vector used for the construction of various vectors containing either wild-type or dominant negative forms of c-*erb*B-2 and c-*erb*B-3. This vector contains an internal ribosome-binding site which allows for the efficient coordinate co-expression of any gene placed into the polylinker region with the neo^r gene located downstream (which together form a single transcription unit when expressed in mammalian cells). (B) Diagramatic representation showing the strategy for blocking wild-type receptor tyrosine kinase function using dominant negative (i.e. truncated) receptors.

in the ends of the c-*erb*B-2 cDNA or the pSLH1001 vector may have occurred during the blunt-end ligations that prevented proper expression. More recent strategies for constructing the required c-*erb*B-2 bicistronic vector now involve the insertion of the c-*erb*B-2 cDNA into the pBKCMV phagemid expression vector (Stratagene) as an intermediary step followed by directional cloning of c-*erb*B-2 into the pSLH1001 vector in a more straight forward manner not involving blunt-end ligations (see below). During the course of this work, we had also employed alternate strategies to derive c-*erb*B-2-overexpressing cell lines, designated MCF-10A*erb*B-2sh (**s**elect **h**igh) and MCF-10A*erb*B-2shH (**s**elect **h**igh **H**igher) cells, that progressively overexpress c-*erb*B-2 at very high levels truly comparable to that seen in breast carcinoma cells with c-*erb*B-2 gene amplification (see previous progress report). Interestingly, while the original MCF-10A*erb*B-2 cells do not show sufficiently high levels of c-*erb*B-2 expression, by using Flow Cytometry selection with anti-p185^{erbB-2} antibody and other selection strategies involving growth factor deprivation we have now successfully generated MCF-10A*erb*B-2-derived cell lines showing very high levels of c-*erb*B-2 gene overexpression, and this allowed us to accomplish the specific aims of the grant (Table 1).

Table 1. ***Derivation of the MCF-10AerbB-2 cell lines****

	MCF-10A	MCF-10A *erb*B-2	MCF-10A *erb*B-2Hyg	MCF-10A *erb*B-2sh	MCF-10A *erb*B-2shH
Origin	Normal reduction mammoplasty tissue	MCF-10A cells infected with pNO2-*erb*B-2 vector	MCF-10A*erb*B-2 cells selected on high levels hygromycin	MCF-10A *erb*B-2 cells after Flow Cytometry	MCF-10A *erb*B-2sh cells cultured w/o growth factors
Infected with vector	no	yes	yes	yes	yes
c-*erb*B-2 gene expression	low	low-moderate	low	moderate-high	high

* See previous project report for details concerning the derivation of these cell lines.

We and others have previously used the MCF-10A normal human mammary epithelial cell line engineered to overexpress c-*erb*B-2 (44, 47). The parental MCF-10A cell line was isolated from normal reduction mammoplasty tissue, and is not tumorigenic in nude mice (54). MCF-10A*erb*B-2 cells were previously infected with a retroviral expression vector containing the human c-*erb*B-2 gene (47). As mentioned above, we have recently confirmed that MCF-10A*erb*B-2 cells do express elevated levels of constitutively tyrosine-phosphorylated p185^{erbB-2} (44). However, they overexpress c-*erb*B-2 at only a fraction of what is measured in mammary carcinoma cells with c-*erb*B-2 gene amplification (unpublished data), and they show very little growth factor independence in culture (44). Although $CdCl_2$ or $ZnCl_2$ was added to cultures in various experiments, the metallothionine promoter that drives c-*erb*B-2 expression in the Hyg^r pNO2*erb*B-2 vector (see Fig. 1 in previous progress report) was found to be very leaky and showed no significant induction in the presence of heavy metals. More recently, however, we noticed a wide range of expression levels of cell surface p185^{erbB-2} in MCF-10A*erb*B-2 cells in experiments utilizing Flow Cytometry to measure cell surface p185^{erbB-2} with FITC conjugated

antibodies directed against p185^{erbB-2} (see Fig. 2 in previous progress report). Different procedures were employed in an effort to select cells with higher levels of c-*erb*B-2 gene overexpression in these heterogenous clones from the original retroviral-infected MCF-10A*erb*B-2 cell population. Strategies for selecting high-level expression of c-*erb*B-2 involved either increasing the selective pressure with titration's of higher levels of hygromycin (i.e. MCF-10A*erb*B-2Hyg cells), direct isolation by Flow Cytometry with antibodies to cell-surface p185^{erbB-2} (i.e. MCF-10A*erb*B-2sh), as well as by the further selection of MCF-10A*erb*B-2sh cells for growth factor independence under growth factor-free, serum-free conditions in culture (i.e. MCF-10A*erb*B-2shH cells). Flow Cytometry selection of MCF-10A*erb*B-2 cell populations with antibodies to cell-surface p185^{erbB-2} was quite effective in deriving a population of cells with significantly elevated levels of p185^{erbB-2} that is truly comparable to that seen in the

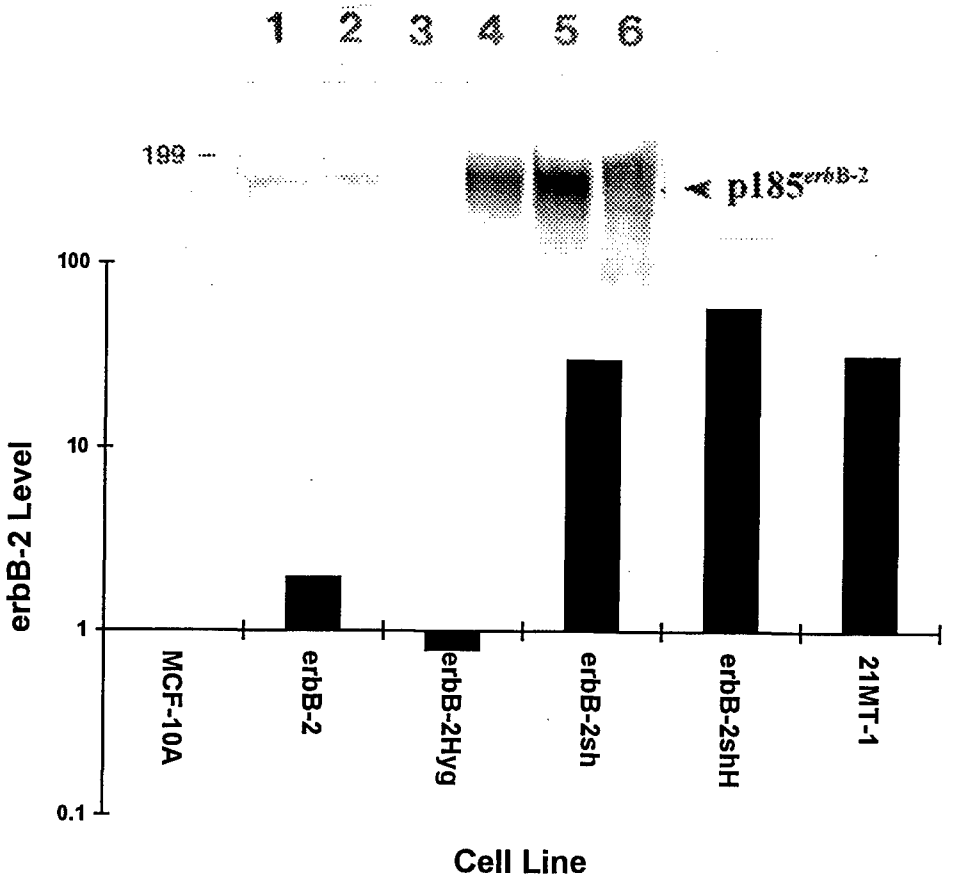


Fig. 2. ***Expression of p185^{erbB-2} by variously selected MCF-10AerbB-2 cell populations.*** Western blot containing equal amounts (100 ug) of total lysate protein per lane were probed with anti-p185^{erbB-2} polyclonal antibody, secondary antibody, strep-avidin-horse radish peroxidase (HRP), and visualization with diaminobenzidine as substrate. MCF-10-A cells (Lane 1); MCF-10A*erb*B-2 cells (Lane 2); MCF-10A*erb*B-2Hyg cells (lane 3); MCF-10A*erb*B-2sh cells (lane 4); MCF-10A*erb*B-2shH cells (lane 5); 21MT-1 breast carcinoma cells (lane 6). Band intensity was quantified by scanning and the levels are shown relative to that measured in the MCF-10A cells.

21MT breast carcinoma cells with c-*erb*B-2 gene amplification (Fig. 2; see also Fig. 3 in previous progress report). In contrast, selection in high levels of hygromycin showed no increase in $p185^{erbB-2}$. Growth factor deprivation increased the levels of $p185^{erbB-2}$ above that seen with Flow Cytometry alone (Fig. 2; see also Fig. 3 in previous progress report). Also, the levels of activated $p185^{erbB-2}$ in both Flow Cytometry-selected cell populations, MCF-10A*erb*B-2sh and MCF-10A*erb*B-2shH cells, was considerably elevated over that seen in the original MCF-10A*erb*B-2 cell population as shown in anti-phosphotyrosine Western blots (not shown). Immunocytochemistry of $p185^{erbB-2}$ levels in the variously selected cell populations (see Fig. 3 in previous progress report) indicated that the selection of growth factor-independent phenotypes by growth factor deprivation leads to a more homogenous population of very high-level c-*erb*B-2-overexpressing cells. As reported in the previous progress report, dramatic alterations in cell morphology were observed for the MCF-10A*erb*B-2sh and MCF-10A*erb*B-2shH cell lines, and these differences were possibly indicative of a more fully transformed phenotype. Therefore, we have now used these MCF-10A*erb*B-2-derived cell lines for further in-depth study of the effects of progressively elevated c-*erb*B-2 gene overexpression on growth factor independence in culture, and tumorigenicity *in vivo*.

The MCF-10A*erb*B-2sh and MCF-10A*erb*B-2shH cell lines were found to grow in the complete absence of IGFs. However, while MCF-10A*erb*B-2sh cells survive and MCF-10A*erb*B-2shH cells grow slowly in the absence of EGF, these cells do not proliferate well in the absence of EGF (Fig. 3). This is in striking contrast to that seen for the 21MT-1 tumor cells

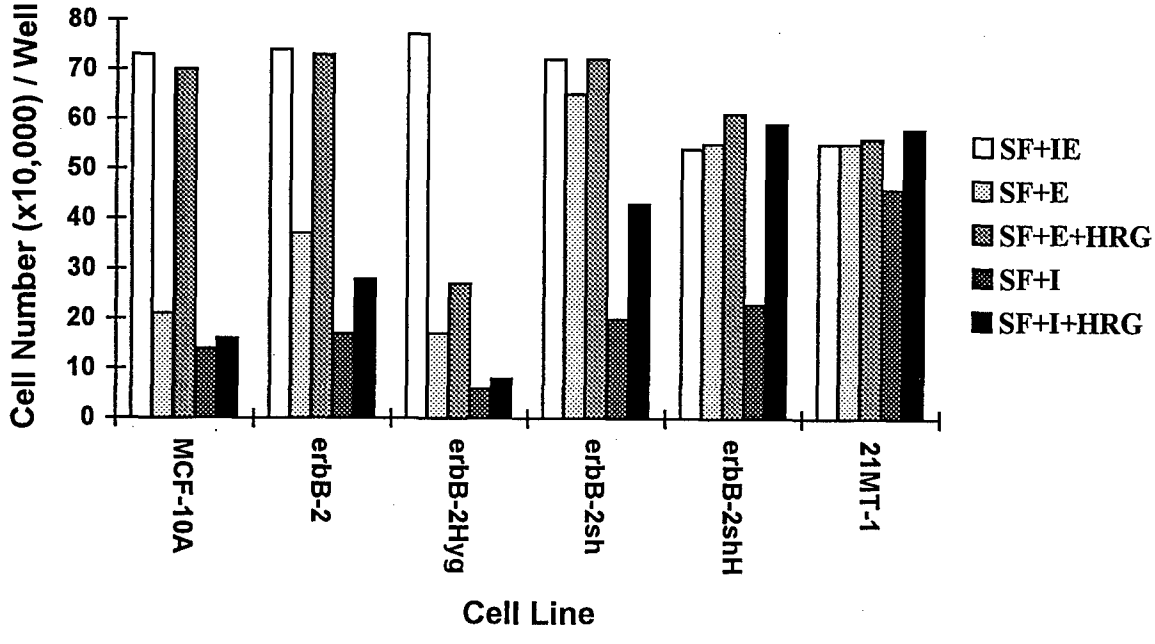


Fig. 3. ***Growth factor independence and HRG-responsiveness of variously selected MCF-10AerbB-2 cell populations in culture.*** Cells were plated at 10^5 cells per well and cultured for 10 days in serum-free (SF) media containing 10ng/ml EGF (E) and/or 5 ug/ml insulin (I) without or with 10 ng/ml HRG-β as previously described (43). The standard deviation was less than 15% in all cases.

with c-*erb*B-2 gene amplification, which are almost completely EGF-independent for growth in monolayer serum-free culture (23). Thus, even very high-level overexpression of $p185^{erbB-2}$ in MCF-10A*erb*B-2shH cells did not make these cells fully EGF-independent under our serum-free culture conditions.

Further experiments were carried out to test for the transformed phenotype of MCF-10A*erb*B-2shH cells both in culture and *in vivo*. MCF-10A*erb*B-2shH cells were found to form foci in monolayer culture (Fig. 4), and to form large colonies in soft agarose culture with high efficiency (Fig. 5). As reported previously (47), MCF-10A*erb*B-2 cells do form some small colonies. However, MCF-10A*erb*B-2 cells form colonies only at a very low efficiency and with small diameters compared to the MCF-10A*erb*B-2shH cells (Fig. 5). This indicates that the quantitative difference in c-*erb*B-2 gene expression between these different lines (i.e. moderate to high levels of $p185^{erbB-2}$) significantly determines the threshold level of signaling required for the effective transformation of normal mammary epithelial cells. Further in-depth quantification of the cloning efficiency and colony diameter for the different cell lines is still in progress.

Preliminary *in vivo* experiments have been carried out using nude mice as recipients. While, as previously reported (47), MCF-10A and MCF-10A*erb*B-2 cells never form tumors in nude mice, MCF-10A*erb*B-2shH cells did form small tumors in nude mice. Early experiments utilyzing direct s.c. injection of cell suspension required over 10^7 cells for tumor formation, but more recent injections in which the cells were embedded in Matrigel matrix showed better tumor formation using lower numbers of cells. Here, 4 of 4 injections formed tumors within a month of injection. More transplants are under way, and some of these tumors are presently being examined by histological and immunohistochemical methods as outlined in the original proposal. Thus, while the original MCF-10A*erb*B-2 cells do not display a significantly transformed phenotype, MCF-10A*erb*B-2shH cells form foci, grow efficiently under anchorage-independent conditions in soft agarose culture, and form tumors in nude mice. This indicates that the high-level of c-*erb*B-2 gene overexpression seen in the MCF-10A*erb*B-2shH cells induces a fully transformed phenotype.

Effects of c-*erb*B-2 Overexpression on PI 3-kinase Activation

Western blotting for p85 of PI 3-kinase in anti-phospotyrosine immunoprecipitates was done to measure the levels of activated PI 3-kinase in cells under growth factor-free conditions, as we have done for other cell lines previously (23). As in the 21MT tumor cell lines with c-*erb*B-2 gene amplification, MCF-10A*erb*B-2sh and MCF-10A*erb*B-2shH cells (but not the MCF-10A*erb*B-2 parental cell population) showed constitutive activation of $p185^{erbB-2}$ (not shown) and PI 3 kinase (Fig. 6), being highest in the MCF-10A*erb*B-2shH cells. Thus, increased levels of PI 3-kinase constitutive activation (i.e. in the complete absence of exogenous growth factors) are associated with the increased levels of $p185^{erbB-2}$ in the MCF-10A*erb*B-2sh and MCF-10A*erb*B-2shH cells, as well as the fully transformed phenotype of the MCF-10A*erb*B-2shH cells.

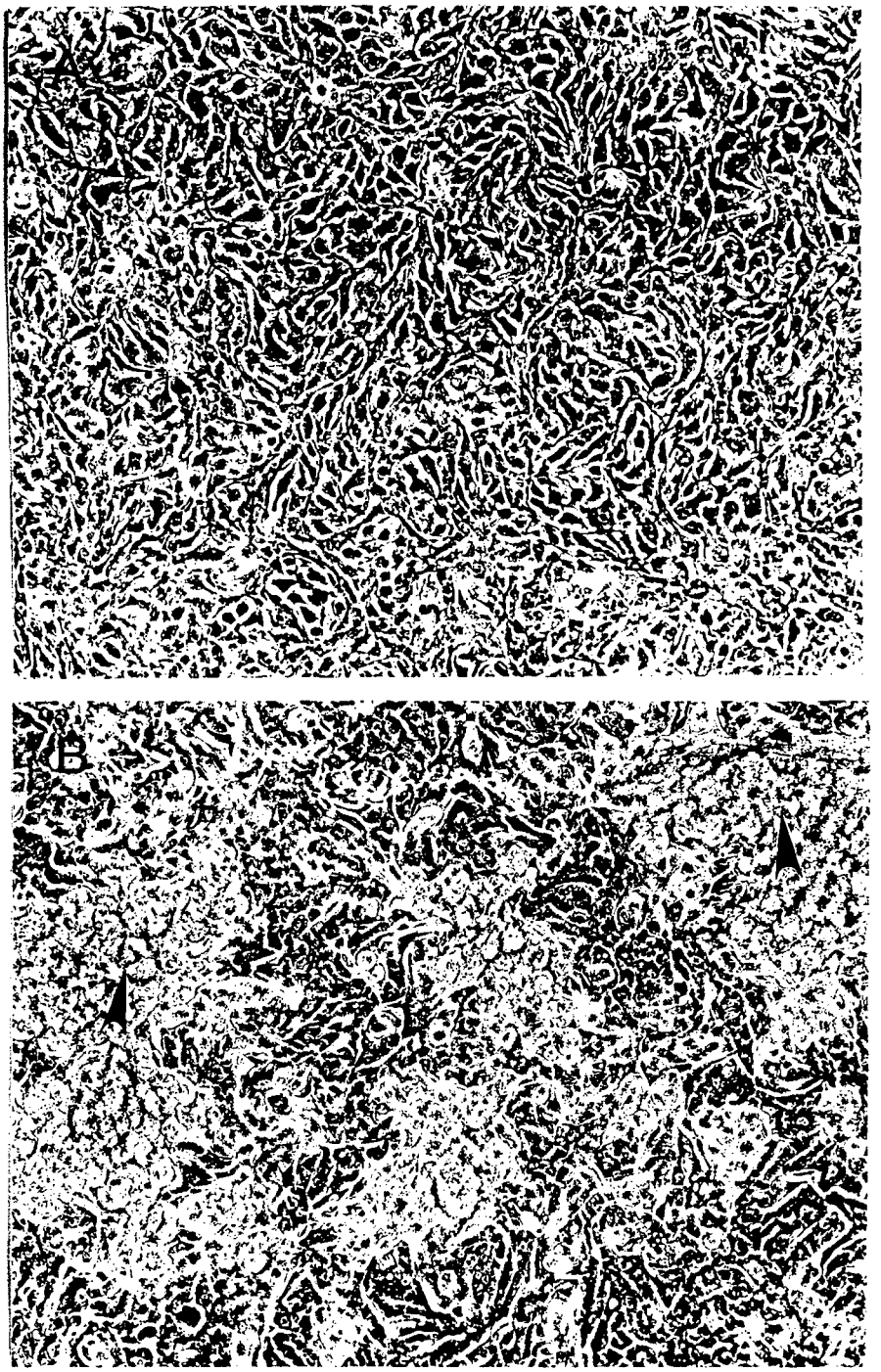
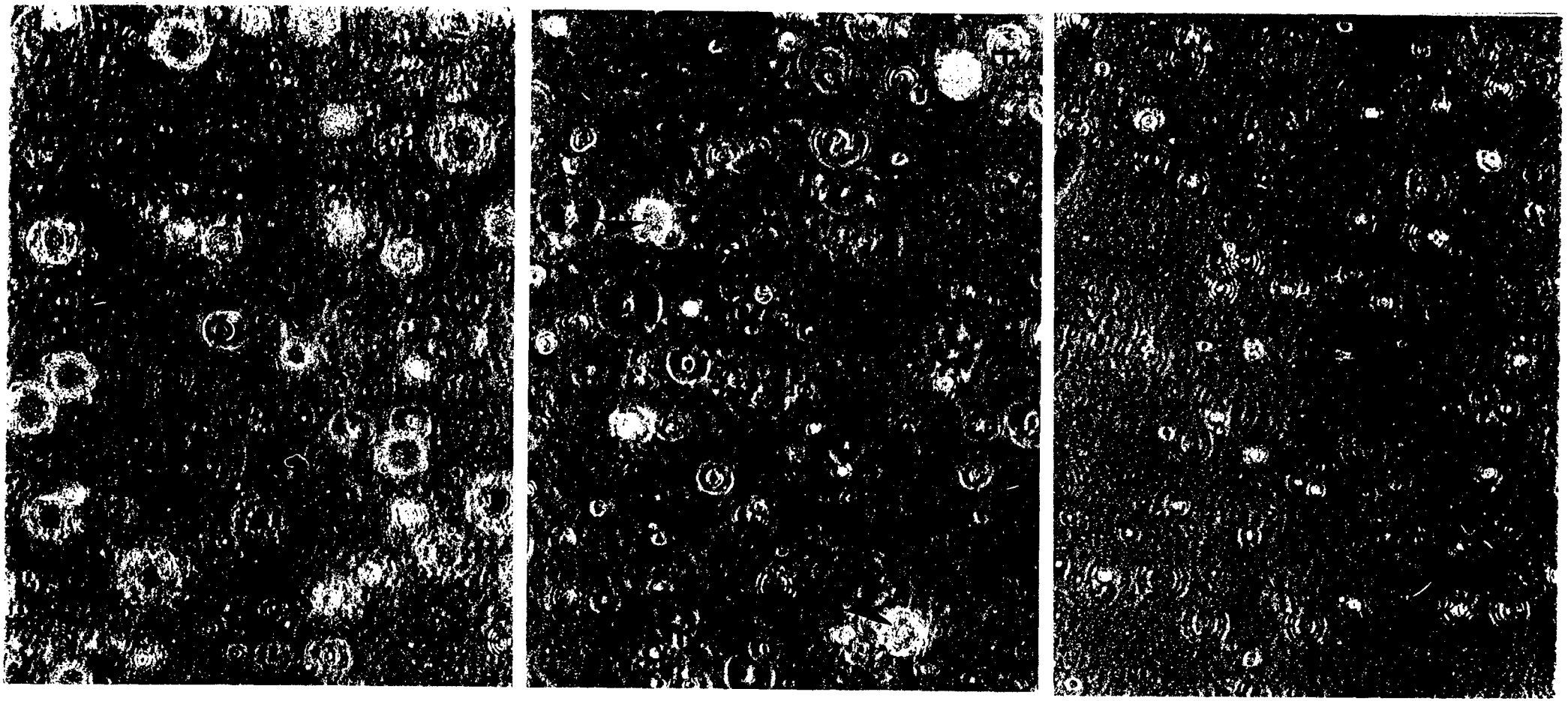


Fig. 4. ***Morphology of MCF-10AerbB-2shH cells in monolayer culture.*** Phase contrast microscopy of MCF-10A*erb*B-2 (A) and MCF-10A*erb*B-2shH (B) cells grown for over a month in monolayer culture, in which time large multicellular foci (arrows) were seen to form for the MCF-10A*erb*B-2shH cells.

(next page)

Fig. 5. ***Anchorage-independent growth of MCF-10AerbB-2shH cells in culture.*** Phase contrast microscopy of MCF-10A*erb*B-2 (A) and MCF-10A*erb*B-2shH (B and C) cells grown for over a month in 0.3% soft agarose culture. The MCF-10A*erb*B-2shH cells form large colonies in soft agarose (B; arrows), and the addition of HRG-β1 (C) greatly augments the colony forming efficiency of the MCF-10A*erb*B-2shH cells as well. 4 mm = 100 um.



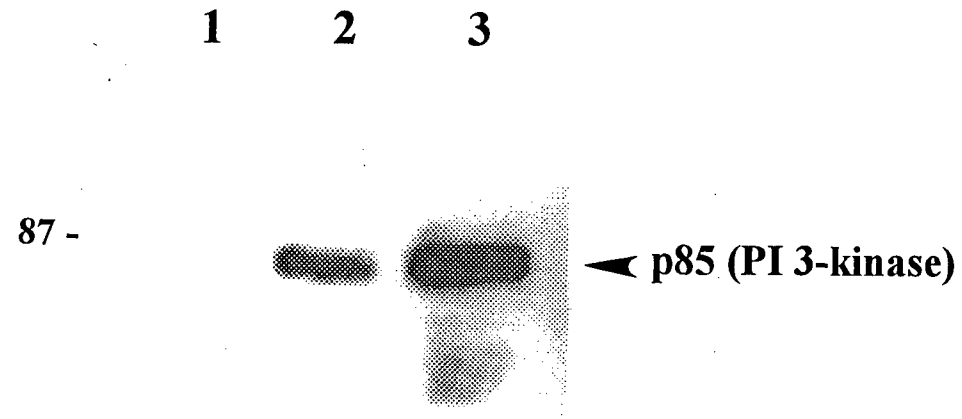


Fig. 6. ***Constitutive activation of PI 3-kinase in MCF-10AerbB-2sh and MCF-10AerbB-2shH cells.*** Cells were incubated in serum-free medium in the complete absence of growth factors for 24 hours prior to the isolation of whole cell lysates and immunoprecipitation (2 mg protein/sample) with anti-phosphotyrosine antibody, followed by Western blotting and probing with anti-p85 antibody, secondary antibody, strep-avidin HRP, and visualization with enhanced chemiluminescent substrate as described previously (23). MCF-10A*erb*B-2 cells (lane 1); MCF-10A*erb*B-2sh cells (Lane 2); MCF-10A*erb*B-2shH cells (Lane 3).

Effects of c-*erb*B-2 Overexpression on Heregulin Expression

It was recently reported that experimentally generated overexpression of c-*erb*B-2 in MCF-10A cells induces higher levels of endogenous HRG expression, and that exogenous HRG induces the anchorage-independent growth of MCF-10A cells (55). Therefore, we were interested to see if the putative autocrine action of HRGs may be involved in the full transformation we observed for the MCF-10A*erb*B-2shH cells, and how responsiveness to exogenous HRGs may vary in our cell line series as a function of progressively increasing levels of $p185^{erbB\text{-}2}$. Preliminary data indicate that under our culture conditions the MCF-10A*erb*B-2 parental cell line did not overexpress HRG cell surface protein relative to the parental MCF-10A cells, however, the MCF-10A*erb*B-2sh and MCF-10A*erb*B-2shH cells did show an increase in the levels of HRG in Western blots containing equal amounts of whole cell lysate protein isolated from the different cell lines in culture (Fig. 7). We routinely detected both ~70 kDa and ~110 kDa bands in cell lysates prepared from these and other cell lines that are known to express HRG. While the previous report of Mincione et al. (55) did apparently detect these same sizes of HRG protein that could be specifically competed out with recombinant HRG peptide, they reported significantly less of the 70 kDa band in the parental MCF-10A cells than what we observed. The only possibly significant quantitative difference we saw was that seen in the MCF-10A*erb*B-2sh and MCF-10A*erb*B-2shH cell lines relative to the parental lines. Thus, the quantitative difference in c-*erb*B-2 gene expression seen in these cell lines with very high-

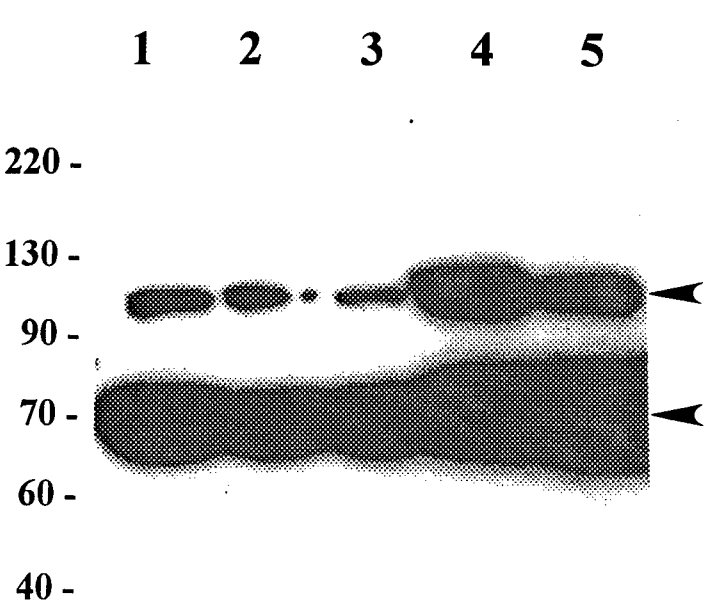


Fig. 7. ***HRG expression by MCF-10A-derived cell lines.*** Western blot containing equal amounts (100 ug) of total lysate protein per lane were probed with anti-HRG monoclonal antibody, secondary antibody, strep-avidin-HRP, and visualization with enhanced chemiluminescent substrate. MCF-10-A cells (Lane 1); MCF-10A*erb*B-2 cells (Lane 2); MCF-10A*erb*B-2Hyg cells (lane 3); MCF-10A*erb*B-2sh cells (lane 4); MCF-10A*erb*B-2shH cells (lane 5).

level c-*erb*B-2 overexpression may induce HRG expression to some extent, and may be critically dependent on a threshold level of c-*erb*B-2 overexpression that is also required for the effective transformation of these mammary epithelial cells. Further experiments are also underway employing recombinant HRG peptide as controls, Northern blotting to measure HRG mRNA expression levels, and to determine which HRG isoforms (i.e. α, β, and or γ) are being expressed by the different cell lines by RT-PCR analysis.

In order to more rigorously test the possibility that autocrine HRG production may be involved in the transformed phenotype of MCF-10A*erb*B-2shH cells, we feel it is necessary to more critically evaluate potential autocrine effects with perturbative strategies employing both antagonistic antibodies to *erb*B-3 as well as the dominant negative *erb*B-3 vectors we have now developed (see below). This type of more critical analysis of potential HRG autocrine loops has not been done previously by other workers in that all reports have, so far, only been correlative. Therefore, we feel that the development and use of strategies which block $p185^{erbB\text{-}2}$/*erb*B-3 activation will be ideally suited to directly test for the possibility of HRG autocrine action, as well as the ligand-independent effects of high-level c-*erb*B-2 overexpression on constitutive activation of signaling pathways and transformed phenotypes (see original proposal for more background concerning these points).

In order to directly study potential HRG-dependent effects on $p185^{erbB\text{-}2}$/*erb*B-3 activation in normal and breast cancer cells, we have began to utilize a recently characterized anti-*erb*B-3 monoclonal antibody (H105) that is a very effective antagonist for HRG binding to *erb*B-3 (59). In pilot studies, we found this antibody to almost completely block the activation of PI 3-kinase

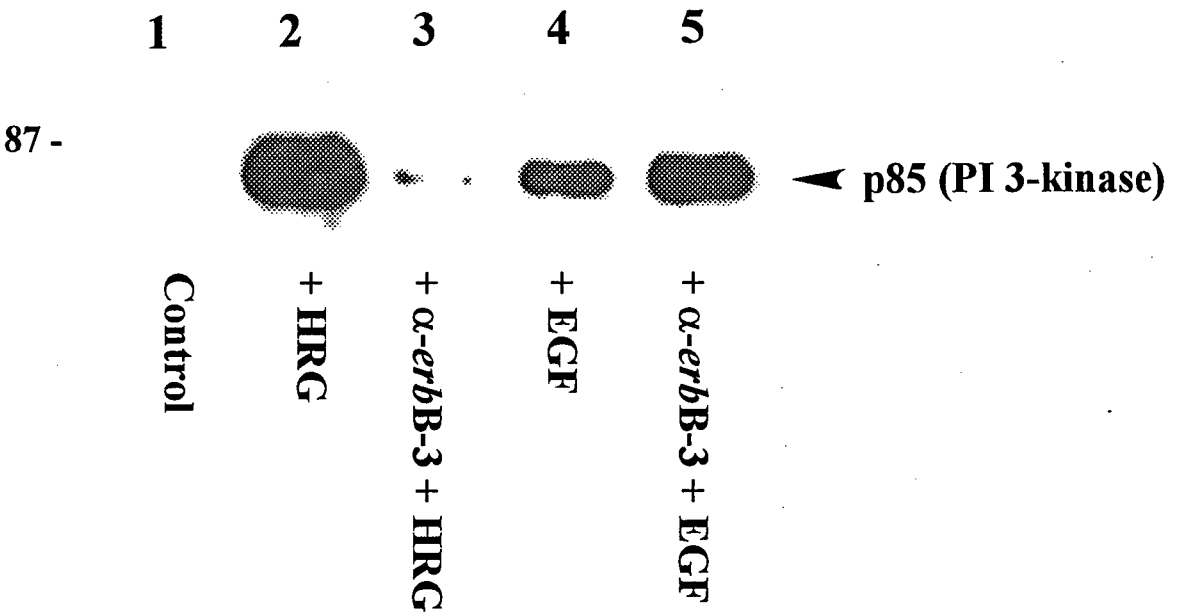


Fig. 8. ***Anti-erbB-3 antagonist monoclonal antibody H105 effectively inhibits the activation of PI 3-kinase by HRG.*** H16N-2 cells were incubated in serum-free medium in the complete absence of growth factors in culture for 48 hrs (Lane 1) prior to the addition of anti-*erb*B-3 monoclonal antibody (Lanes 3 and 5) for 1 hour, followed by stimulation of cells with 10 ng/ml HRG-β1 (Lanes 2 and 3) or EGF (Lanes 4 and 5) for 10 minutes at 37° C. Whole cell lysates (2 mg protein/sample) were then prepared and immunoprecipitated with anti-phosphotyrosine antibody, followed by Western blotting and probing with anti-p85 antibody, secondary antibody, strep-avidin-HRP, and visualization with enhanced chemiluminescent substrate.

by exogenous HRG in H16N-2 cells (Fig. 8). Interestingly, this antibody did not block the EGF-induced activation of PI 3-kinase. It is known that most of the PI-3 kinase activated by EGF is due to the direct recruitment of PI 3-kinase by *erb*B-3 in EGFR/*erb*B-3 heterodimers in different cancer cell lines (34,35). We have also recently confirmed that this is the case in the H16N-2 cells (which were isolated from normal human mammary epithelium that expresses low, but functional levels of EGFR, $p185^{erbB-2}$ and *erb*B-3) by separately immunoprecipitating the different *erb*Bs after stimulation with either EGF or HRG (manuscript in preparation). An important question then arises concerning the possible ligand-dependency of *erb*B heterodimer activation in which the stimulating ligand only binds directly to one of the kinases within the pair (e.g. when EGFR/*erb*B-3 is activated by EGF). Our result with this anti-*erb*B-3 antibody (Fig. 8) now indicates that the activation of PI 3-kinase by EGF in mammary epithelial cells (which express c-*erb*B-3 but not c-*erb*B-4; 43) does not involve a detectable level of autocrine HRG action concurrent with stimulation of EGFR/*erb*B-3 heterodimers by EGF. Furthermore, we can now use this antibody to directly test for the presence of functional HRG autocrine loops in cancer cells containing constitutive activation of $p185^{erbB-2}$/*erb*B-3. We have already begun to use this antibody for such studies with the 21MT-1 cells, as well as with MDA-MB-231 breast carcinoma cells, which highly overexpress HRGs and may have a functional HRG autocrine loop (manuscript in preparation). These studies will now be extended to use the MCF-10A*erb*B-2-

derived lines for analysis of potential ligand-dependency in the constitutive activation of p185^{erbB-2}/*erb*B-3 in the MCF-10A*erb*B-2shH cells.

Effects of c-*erb*B-2 Overexpression on Growth Factor Deprivation-induced Apoptosis

One interesting observation that was made following the Flow Cytometry selection of MCF-10A*erb*B-2 cells. It was noticed that, while MCF-10A and MCF-10A*erb*B-2 cells die off in the absence of all growth factors in culture, many MCF-10A*erb*B-2sh cells survived after the withdrawal of all growth factors under serum-free conditions in culture. This then allowed us to culture these cells for an extended period in the complete absence of growth factors under high cell density culture conditions (see previous progress report). After confluent cultures were left a month in the absence of all growth factors, these cells began to develop foci-like structures (see Fig. 5 in previous progress report), and these cultures were then passaged at a low split ratio in the absence of any growth factors. As mentioned above, these MCF-10A*erb*B-2shH cells selected in the absence of growth factors showed a more homogenous high-level c-*erb*B-2 expression than did the original Flow Cytometry-selected MCF-10A*erb*B-2sh cell population (see Fig. 3 in previous progress report). This suggested that the selection of these c-*erb*B-2-overexpressing cells in the absence of growth factors resulted in the preferential survival of the highest-level overexpressing cells within the original cell population. As mentioned above, both MCF-10A*erb*B-2sh and MCF-10A*erb*B-2shH cells showed a very high level of IGF-independent growth in culture, while MCF-10A*erb*B-2shH cells grow slowly in the absence of EGF. We were interested in investigating the possibility that the higher levels of c-*erb*B-2 gene expression seen in the MCF-10A*erb*B-2sh and MCF-10A*erb*B-2shH cells (compared to the original MCF-10A and MCF-10A*erb*B-2 cells) specifically prevented apoptosis in the complete absence of IGFs and EGF, as well as a high level of proliferation in the complete absence of IGFs. It has been shown previously (56) that MCF-10A cells, like most normal cells, undergo apoptosis in the absence of IGFs and EGF as assessed by DNA laddering. Our preliminary experiments have now confirmed this, and the MCF-10A*erb*B-2sh and MCF-10A*erb*B-2shH cells did not show detectable DNA degradation in the complete absence of growth factors in culture (not shown). Further experiments are underway involving DNA laddering and alternative apoptosis assays to expand this novel observation, and investigate the possiblity that high-level c-*erb*B-2 overexpression is sufficient to prevent growth factor deprivation-induced apoptosis in MCF-10A cells.

Construction of New *erb*B wild-type and Dominant Negative Vectors for Expression in Normal- and Tumor-derived Cell Lines

In order to accomplish the additional specific aims for this project, it is necessary to construct dominant negative vectors for c-*erb*B-3 and c-*erb*B-2 (see original proposal). Constructing the dominant negative vector for c-*erb*B-3 is an especially important focus for this project, because this has not been previously attempted, and may be especially effective in blocking the heterodimer interactions between p185^{erbB-2} and *erb*B-3. As originally outlined in

the project proposal, we attempted to clone various PCR-generated fragments of c-*erb*B-2 and c-*erb*B-3 cDNAs into the pSLH1001 bicistronic retroviral vector. While the amplification of the 2 kb regions of reverse-transcribed mRNAs was accomplished successfully using a high-fidelity PCR kit (Boehringer Mannheim), these fragments could not be ligated into the Xho I and Cla I restriction sites in the pSLH1001 vector. The primers used to generate these fragments were designed to also contain compatible sites (i.e. Sal I and Cla 1) in the proper orientation for ligation into the pSLH1001 vector (see original proposal). However, during the course of these experiments, it was noted that Sal I sites (which are compatible with the Xho I site in pSLH1001) are known to be only inefficiently cleaved when located in the terminal region of linear DNA fragment. Therefore, we attributed the inability to derive ligation products in these reactions (which also included positive control reactions) to the lack of proper restriction digestion of the PCR-generated fragments prior to ligation. More recently, we have successfully introduced PCR-generated fragments coding for different forms of c-*erb*B-2 and c-*erb*B-3 into pSLH1001 using alternate primers. However, we were not able to detect the expression of the dominant negative proteins in recipient cell lines using these previously constructed vectors. One serious obstacle to strategies for constructing expression vectors with PCR-generated fragments may involve the deleterious mutations that occur at a significant frequency in amplified products, even in protocols using high-fidelity Pou/Taq polymerase enzyme combinations.

An alternate approach for developing the dominant negative expression vectors has now been employed using the available full-length cDNAs (which were only more recently available for c-*erb*B-3). By using the pBK-CMV phagemid expression vector (Stratagene) as an intermediate, we have cloned both wild-type and dominant negative c-*erb*B-3 into the pSLH1001 bicistronic retroviral expression vector using flanking restriction sites located within the extensive polylinker region of pBK-CMV. When the cDNA that we have for c-*erb*B-3 was cleaved with Hind III/Bal I and Sal I/Bam HI, respectively, 2.4 kb and 2.2 kb fragments lacking most of the cytoplasmic domains of these genes were generated that were then ligated into the Hind III/Sca I and Sal I/Bam HI sites of pBK-CMV (Fig. 9). These ligations also introduced inframe stop codons downstream of the point of ligation. These pBK-CMV-derived vectors were then used directly in experiments in which the vectors were transfected into the the H16N-2 and 21MT-1 cell lines. These cells are presently being analyzed for their level of wild-type and dominant negative *erb*B-3 expression, and experiments are underway to derive cell clones from these cell populations for further analysis. For introduction of the vectors into various cell lines that do not show reasonably high transfection efficiencies with antibiotic resistance and marker gene co-expression when transfected with standard monocistronic vectors (Fig. 10; manuscript in preparation), newly constructed bicistronic retroviral expression vectors will be highly useful in that they show 100% co-expression of antibiotic resistance with marker gene expression, and thus eliminates the occurrence of false positive clones. Therefore, it is our ultimate goal to derive cell populations infected with the retroviral vectors. The wild-type and dominant negative forms of c-*erb*B-3 have just been successfully cloned into pSLH1001 by ligation of Sal I/Cla 1-cut inserts (which do not contain these sites internally) isolated from pBK-CMV-derived vectors into the Xho I/Cla I sites (i.e. Sal I and Xho I have compatible ends) of pSLH1001 (Fig. 1). Restriction digests have confirmed the proper construction of these vectors and H16N-2 and 21MT-1 cells have recently been infected with these retroviral vectors (not shown), and cells are

Lane	Enzyme	------- Predicted fragment length (bp) ------- pBK-3	pBK-3dn
1	Cla I	9460	6788
2	Hind III	5435, 2354, 1671	5117, 1671
3	Sal I	9460	6788
4	Cla I / Hind III	5010, 2779, 1671	5010, 1671, 107
5	Cla I / Sal I	5009, 4451	4451, 2337
6	Hind / Sal I	4876, 2354, 1671,559	4558, 1671, 559

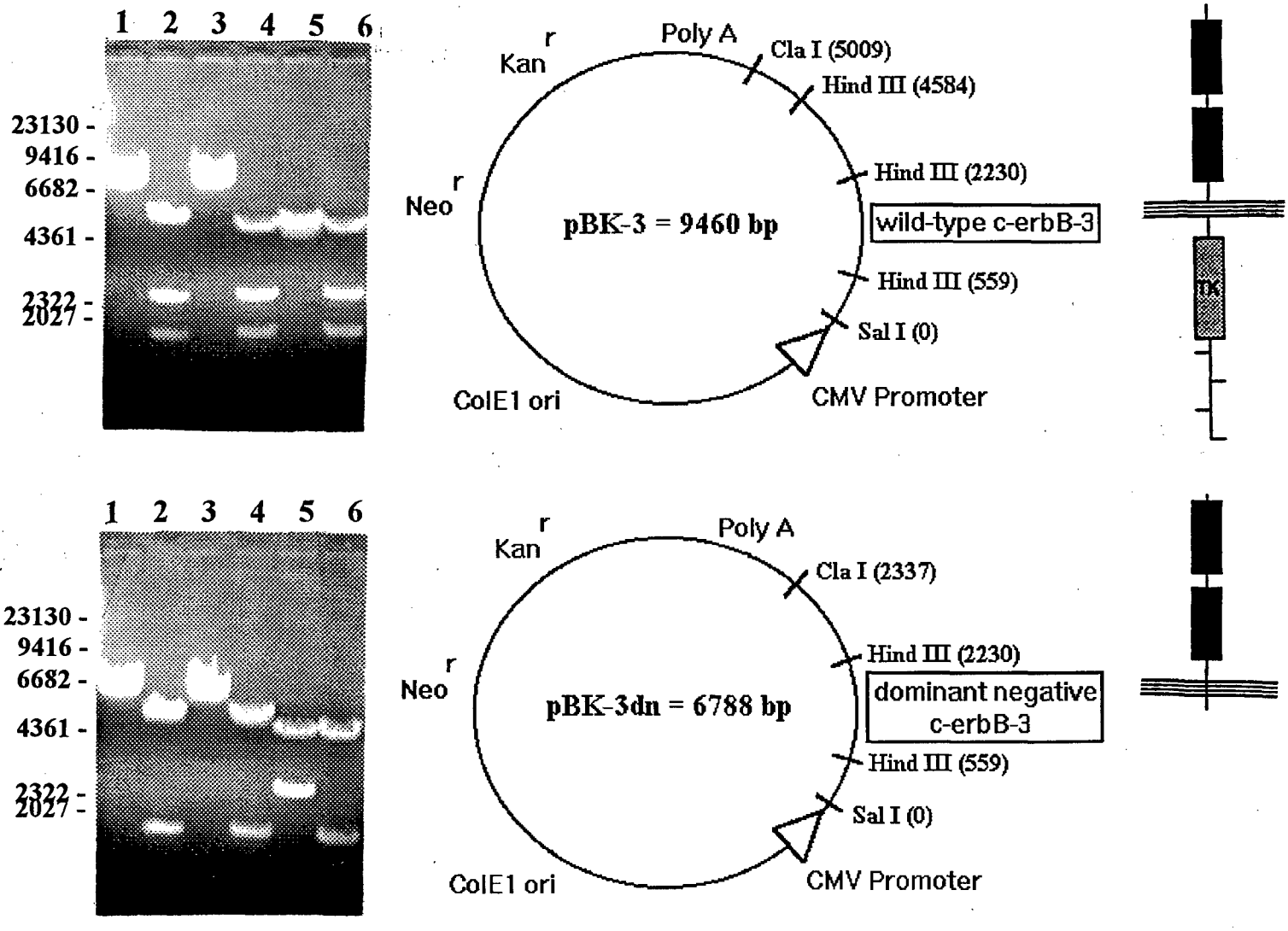


Fig. 9. ***Restriction digest analysis of c-erbB-3-derived vectors.*** Extensive restriction digest analysis was performed on all vectors mentioned in the text as is shown here for the pBK-3 and pBK-3dn vectors. These vectors are presently being used for transfection experiments, and were used as intermediates for the construction of the bicistronic retroviral expression vectors, pSLH-3 and pSLH-3dn, containing wild-type and dominant negative forms of c-*erb*B-3.

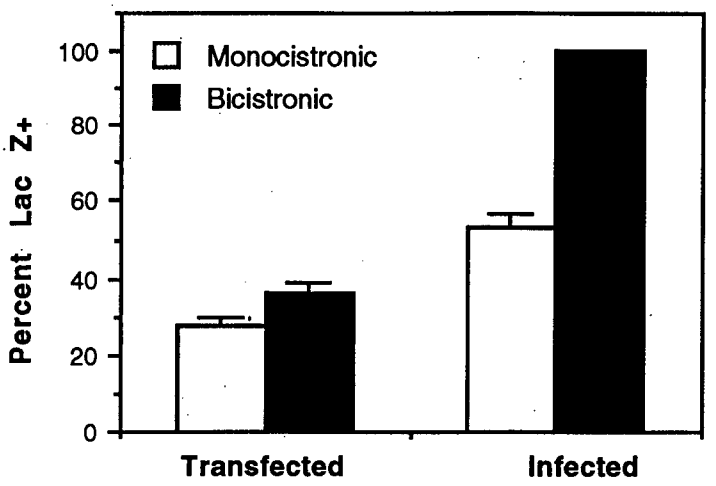


Fig. 10. ***Co-expression of marker genes with neomycin resistance in cells stabiliy transfected or infected with monocistronic or bicistronic retroviral expression vectors.*** H16N-2 cells were either transfected or infected with two different retroviral expression vectors: one monocistronic (i.e. containing the Lac Z and neo^r genes on separate transcription units under the control of separate promoters), and the other bicistronic (i.e. containing both the Lac Z and neo^r genes on a single transcription unit containing an internal ribosome-binding site). After selection for one month on G418, cell colonies were stained for β-Galactosidase activity, and the number of blue and white colonies were counted to determine the efficiency of Lac Z/neo^r gene co-expression. Notice that 100% co-expression is achieved when bicistronic retrovral vectors are introduced into cells by infection, thus eliminating the problem of false positives arising during the antibiotic-resistance selection of cells genetically engineered to ectopically express a gene-of-interest. The mean average and standard deviation is shown.

presently being selected on G418 for further analysis. These cells will be invaluble for completing the specific aims of the grant.

Further ligations will be required for the construction of c-*erb*B-2-derived vectors as previously outlined (see previous progress report). Unsuccessful ligations for the c-*erb*B-2-derived vectors may be due to the use of the Bal I site for ligation into pBK-CMV, which generates blunt ends rather than overlapping compatible ends as was possible for both ends of the c-*erb*B-3-derived vectors. Therefore, an alterative approach has been devised for the c-*erb*B-2-derived vectors utilyzing the pcDNA (In Vitrogen) cloning vector as an intermediate. The Hind III/Sal I fragment of the full-length c-*erb*B-2 cDNA will be first ligated into the polylinker region of pcDNA. A Hind III/Xho I flanking *erb*B-2 insert will then be cloned directly into pBK-CMV, and cutting the vector with Sac I followed by recircularization will generate a dominant negative form of c-*erb*B-2 with a termination codon in frame and downstream of the insert. Sal I/Cla 1-cut *erb*B-2 inserts (which also do not contain these sites internally) isolated from pBK-CMV-derived vectors will then be cloned into pSLH1001 as previously outlined.

CONCLUSIONS

We have now successfully produced MCF-10A-derived cell populations that overexpress c-*erb*B-2 at very high levels comparable to that seen in breast carcinoma cells with c-*erb*B-2 gene amplification. These and the other cell lines presently under construction will be invaluable for fulfilling the specific aims of this grant (see original proposal). While the original clones of MCF-10A*erb*B-2 cells overexpress c-*erb*B-2 at only moderate levels, very high-level overexpression of c-*erb*B-2 was obtained by selecting cells from the original heterogenous MCF-10A*erb*B-2 cell population using Flow Cytometry with anti-$p185^{erbB-2}$ antibody. Following Flow Cytometry selection of c-*erb*B-2-overexpressing (i.e. MCF-10A*erb*B-2sh) cells, these cells were then further selected in the complete absence of growth factors in serum-free culture, and this further increased the levels of $p185^{erbB-2}$ in the resulting MCF-10A*erb*B-2shH cells. Experiments are continuing to study the changes in the levels of $p185^{erbB-2}/p185^{erbB-2}$ and $p185^{erbB-2}$/*erb*B-3 constitutive activation seen in these cell lines, and to relate the accompaning changes in PI 3-kinase activation (and other effects of c-*erb*B-2 overexpression) to the threshold level of signaling sufficient for the effective transformation of mammary epithelial cells.). In addition, key observations were made during the course of these studies which provide important information concerning the survival and growth of cells in the absence of growth factors that occurs as a function of the level of c-*erb*B-2 gene overexpression.

Interestingly, previous studies employing NIH 3T3 as a recipient cell line initially reported that the wild-type c-*erb*B-2 was not oncogenic. However, subsequent work showed that these earlier studies had not properly tested the oncogenic potential of the wild-type c-*erb*B-2 gene, because the levels of c-*erb*B-2 overexpression was not high enough to constitutively activate $p185^{erbB-2}$ to levels sufficient to transform NIH 3T3 fibroblast cells (24-26). By using different promoters and the DHFR replicon in newly developed expression vectors, subsequent researchers were able to generate cell lines which overexpress very high levels of $p185^{erbB-2}$, and were able to properly test the oncogenic potential of the wild-type c-*erb*B-2 gene in NIH 3T3 cells (24-26). In those studies, it was apparent that a level of $p185^{erbB-2}$ of approximately $5x10^5$- $8x10^6$ receptors/cell were required for transformation mediated by $p185^{erbB-2}$ homodimers (i.e. NIH 3T3 cells do not express significant levels of the other *erb*Bs). As mentioned above, more recent studies have now also demonstrated a cooperative transforming capability between *erb*B-3 and $p185^{erbB-2}$ in NIH 3T3 cells (22). However, it is still not clear how the presence of even low levels of *erb*B-3 quantitatively affects the critical threshold level of $p185^{erbB-2}$ required for the effective transformation of normal human mammary epithelial cells. The studies that have tested the oncogenic potential of wild-type c-*erb*B-2 overexpression in human mammary epithelial cells are likely to be directly analogous to that seen earlier using NIH 3T3 cells, in that reports of the ability of c-*erb*B2 to fully transform MCF-10A cells have not yet been adequately tested due to the difficulty of generating cell lines that overexpress sufficiently high levels of $p185^{erbB-2}$. We have now made major progress towards resolving this issue by deriving MCF-10A*erb*B-2shH cells that do overexpress c-*erb*B-2 at levels comparable to that seen in breast carcinoma cells with c-*erb*B-2 gene amplification. Furthermore, the ability of MCF-10A*erb*B-2shH cells to grow efficiently under anchorage-independent growth conditions in culture and to form tumors in nude mice indicates that the high-level of c-*erb*B-2 gene expression seen in these cells results in a completely transformed

phenotype, which was not seen in the original MCF-10A*erb*B-2 cells that only show a moderate level of c-*erb*B-2 gene overexpression. We conclude that the threshold level of c-*erb*B-2 gene expression sufficient for effective transformation of MCF-10A cells is between that seen in the MCF-10A*erb*B-2 and MCF-10A*erb*B-2shH cell populations. Flow Cytometry-selected MCF-10A*erb*B-2sh cells were found to survive in the absence of any exogenous growth factors under high density culture conditions, and these cells were then cultured for extensive periods under selective pressure in the absence of exogenous growth factors. This selective pressure was found to lead to a more homogenous distribution of high-level c-*erb*B-2 overexpressing cells (see previous project report). Furthermore, these experiments in addition to others (manuscript in preparation) support the contention that there is an important relationship between growth factor independence and the progressively increasing levels of c-*erb*B-2 gene expression seen in breast carcinoma cells with c-*erb*B-2 gene amplification (43). By using MCF-10A-derived cell lines which co-express *erb*B-3, we can now directly test the effects of *erb*B-3 cooperativity in genetically engineered normal human mammary epithelial cells that overexpress sufficiently high levels of $p185^{erbB-2}$ for transformation.

As discussed in the original grant proposal and above, while HRG expression occurs in some breast cancers, the potential role of HRG autocrine action in the constitutive activation of $p185^{erbB-2}$/*erb*B-3 in different breast cancer cell lines has not been more critically assessed by direct perturbative analysis. We can now use HRG antagonist anti-*erb*B-3 antibody H105 to directly test for the presence of functional HRG autocrine loops in cancer cells containing constitutive activation of $p185^{erbB-2}$/*erb*B-3. We have already begun to employ this antibody for such studies using the 21MT-1 cells as well as MDA-MB-231 cells, which are breast carcinoma cells known to overexpress HRGs and may have a functional HRG autocrine loop (manuscript in preparation). All of the MCF-10A-derived and 21N/21T cell lines (i.e. H16N-2, 21PT, 21MT-2 and 21MT-1) express low, but detectable levels of HRG. However, it is not yet clear how functionally significant such levels may be, since exogenous EGF or HRG is still required for the proliferation of most of these cell lines in culture (43, 44). Therefore, a more critical analysis using blocking antibodies would be very useful for helping resolve this important issue concerning the potential role of autocrine HRG action in the constitutive activity of $p185^{erbB-2}$/*erb*B-3 in breast cancer cells. These studies will now also be extended by using the MCF-10A-derived cell lines for analysis of potential autocrine activation of $p185^{erbB-2}$/*erb*B-3. This work should also intersect well with other blocking stratagies using the dominant negative expression vectors.

It has been reported that human breast carcinoma cells sometimes overexpress c-*erb*B-3, and it has been suggested that this may be important for the malignancy of certain breast tumors (2). However, while c-*erb*B-3 is commonly expressed at a low but functional level in most normal and transformed mammary epithelial cell lines, c-*erb*B-3 is never appears to be amplified or highly overexpressed. Furthermore, when high levels of c-*erb*B-3 overexpression are genetically enginered in cell lines, this is not sufficient to constitutively activate *erb*B-3 in-of-itself or to transform cells (22). By itself, *erb*B-3 is a very weak kinase compared to the other erbBs (5). Rather, *erb*B-3 is constitutively activated only in cell lines in which cooperative interaction with $p185^{erbB-2}$ activates *erb*B-3 in $p185^{erbB-2}$/*erb*B-3 heterodimers (22). This is most often seen in cell lines which highly overexpress c-*erb*B-2 due to gene amplification in cancer cells, or experimentally by introduction of c-*erb*B-2 expression vectors containing strong

promoters (22, 23, 43). Interestingly, all of the cell lines we use for our studies do not overexpress c-*erb*B-3 relative that seen in comparable normal cell lines (unpublished data). Therefore, it is our contention that even low levels of *erb*B-3 cooperate with p185 $^{erbB-2}$ to effectively transform breast carcinoma cells with c-*erb*B-2 amplification, but this mechanism of cell transformation does not necessarily require concordant overexpression of c-*erb*B-3. This hypothesis can be directly tested by overexpressing c-*erb*B-3 in the different c-*erb*B-2 overexpressing cell lines (that now only express low, but functional levels of c-*erb*B-3) in order to determine if elevated levels of c-*erb*B-3 gene expression in these cell lines augment the tumorigenic or metastatic potential of breast cancer cells with c-*erb*B-2 gene amplification. Interestingly, the c-*erb*B-3 wild-type vectors that we have already constructed will now also allow us to directly test this hypothesis by deriving c-*erb*B-3-overexpressing 21PT, 21MT-2 and 21MT cell lines. These cells will then be analyzed to assess their relative degree of p185^{erbB-2}, *erb*B-3 and PI 3-kinase activation, as well as their colony forming efficiency in culture, and their tumorigenic potential *in vivo*.

It was recently reported that p53-independent apoptosis is induced in MCF-10A cells in response to IGF and EGF deprivation in culture (56). Their study also showed that the actions of the MAP-kinase and PI 3-kinase pathways were required to prevent apoptosis in non-neoplastic mammary epithelial cells (56). Our results now also suggest that the constitutive activation of p185^{erbB-2}, *erb*B-3 and PI 3-kinase induced by high-level wild-type c-*erb*B-2 overexpression promotes cell survival in the absence of exogenous growth factors in culture. Further study of the minimum level of c-*erb*B-2 gene overexpression required to induce these effects will allow us to better understand the *pleiotropic effects* of c-*erb*B-2 overexpression during mammary tumor progression. In addition, recent studies now also indicate that c-*erb*B-2 overexpression combined with p53 inactivation is correlated with poorer prognosis than c-*erb*B-2 overexpression alone in breast cancer patients (57). This may have important implications regarding the cooperative effects of tumor suppressor gene inactivation with c-*erb*B-2 gene amplification during tumor progression. As outlined in the original proposal, we are also constructing c-*erb*B-2-overexpressing H16N-2 cells. The H16N-2 cells, which were isolated from the same patient as the 21MT cell lines by transfection of normal mammary epithelium with the human papilloma virus (HPV)-16, will provide an independent test of the oncogenic potential of c-*erb*B-2 overexpression in non-neoplastic human mammary epithelial cells that co-express c-*erb*B-3 (43). By using this cell line with p53 and RB inactivation induced by the E6 and E7 gene products, we can compare the transforming potential of p185^{erbB-2} in mammary cells with specific suppressor gene inactivations (i.e. the MCF-10Acells contain functional p53 and RB; 56). We have also recently constructed MCF-10A cells that express the E6 and E7 genes separately, and these cell lines may also prove valuable for later studies comparing the oncogenic potential of p185^{erbB-2} and *erb*B-3 in cells containing various alterations in specific suppressor gene functions.

A major focus of this project involves the construction and use of cell lines expressing dominant negative forms of c-*erb*B-3 and c-*erb*B-2. This will allow us to complete the specific aims of the grant that attempts to inhibit p185^{erbB-2}/*erb*B-3 heterodimer function. The strategy that has now been used successfully for construction of c-*erb*B-3-derived vectors utilizing the full-length cDNA and the splicing together of different cloning vectors. The c-*erb*B-3-derived vectors are presently being used for the extensive analysis of p185^{erbB-2} and *erb*B-3 activation in

the H16N-2 and 21MT-1 cell lines. The use of these and other vectors presently under construction will allow us to successfully complete the specific aims of the project that attempts to inhibit p185^{erbB-2}/*erb*B-3 heterodimer function in cells that overexpress c-*erb*B-2. These studies will then also be extended to study the potential autocrine function of HRGs produced by other breast carcinoma cell lines, such as the MDA-MB-231 cells, as well as the MCF-10A*erb*B-2-derived cell lines.

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APPENDICES

None included.